Qdots technology for biodetection available at LLNL

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In the following note, I will present the qdots technology available at LLNL with a special emphasis on potential applications for biotetection and biolabeling. I will outline the progresses made over the past few years in their synthesis and characterization. I will present the different biomolecules that we have linked to the qdots, particularly single stranded DNA and nuclear localization sequences. This will lead us to the discussion of proof-of-concept experiments focused on pathogen detection and genetic studies using qdots. In the last part, I will outline four lines of research for the near future that cover issues from the synthesis of dual-function probes for MRI and fluorescence detection, to single nanoparticle photophysical properties, nanocrystal toxicity and pure biology.

1. Existing Technology available at LLNL: Water-soluble Qdots

Qdots are core/shell CdSe/ZnS nanocrystals of a few nanometers in size. The fluorescence emission depends on their size, which can be tuned to cover the whole visible spectrum. Compared the organic dyes, qdots are photochemically very robust and every emission color can be excited with the same wavelength. Because qdots are synthesized in organic solvents, they are not water-soluble and therefore useless as such for any type of biotetection scheme. However, it is possible to modify their surface to make them hydrophilic. Currently, there are many methods to impart hydrophilicity to colloidal qdots, but I will focus only in one of them, namely silanization. It consists in embedding the qdots in a polymerized silica shell. The shell is about 1-3 nm thick and its surface can be easily tailored to have thiols, amines, carboxylic, and/or aldehyde groups. Moreover, the silica shell provides an ideal scaffold for doping the particles with MRI (Magnetic Resonance Imaging) active compounds. Silanization of qdots is by far the most versatile methods to solubilize qdots. The dots are soluble in high ionic strengths buffers and for pH values ranging from 4 to 11. As a result, silanized qdots have been already used in various biolabeling experiments including for the detection of viral and bacterial pathogens and nuclear targeting.

2. Conjugation of biomolecules to qdots

Unlike any other water-soluble dots, silanized qdots have thiols at their surface and this makes them unique for bioconjuagtion. Indeed, among the functional groups available (thiols, amines, carboxylic groups, aldehydes), thiols are by far the easiest to modify. Reaction of thiols with maleimides close to neutral pH produces stable linkages. As depicted in Fig.1, we have used this simple reaction scheme to attach small biomolecules (biotins), proteins (streptavidin), peptides, antibodies and single stranded DNA to the qdots.

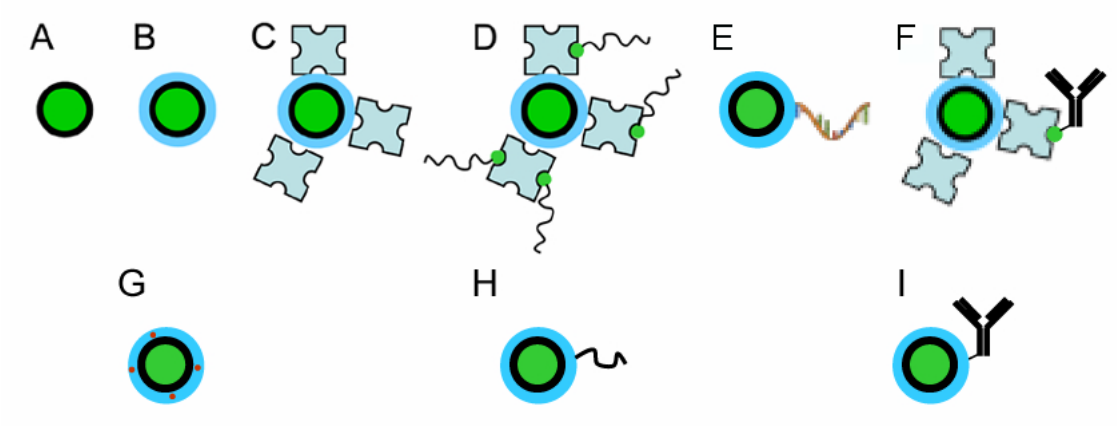

Figure 1. On the top row, there is the scheme of different types of qdots produced in our laboratory. The schemes are not to scale. ***A)*** Core/shell CdSe/ZnS nanocrystals or qdots. ***B)*** Silica (blue) coated qdots. ***C)*** Streptavidin coated qdots. ***D)*** Peptide coated qdots through a streptavidin-biotin bridge. ***E)*** Single stranded DNA-qdot conjugates with direct covalent linkage. ***F)*** Antibody-qdots through a streptavidin-biotin bridge. On the bottom, there is the schematic of possible qdots that we intend to develop. ***G)*** Dual-probe with doped silica. These probes may be MRI active and fluorescent. ***H)*** Peptide-qdots with direct covalent linkage and ***I)*** Antibody-qdots with direct linkage. Probe H and I will have the same functionality than probes in E and F respectively, by will be about 5-7 nm smaller due the bypass of the streptavidin-biotin bridge.

The linking of the biomolecules to the qdots is probed by mobility shifts in gel electrophoresis (Fig.2). Therefore, we have a palette of biomodified qdots available. We believe we can devise different routes to link any biomolecule to the silanized qdots. For instance, we have linked peptides to qdots through a streptavidin-biotin bridge. However, it is possible to link the peptide directly to the qdot through an amide bond. This will produce a smaller structure that may be of interest for nuclear targeting.

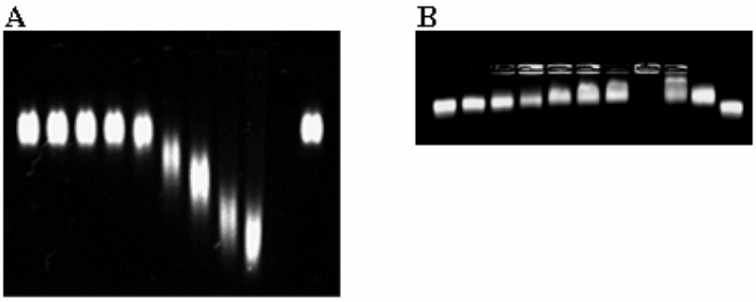

Figure 2. Electrophetic mobility of biomodified qdots. ***A)*** Single-stranded DNA (ssDNA, 21 bases) modified qdots display an increased mobility as the average number of ssDNA per qdot increases (Lane1 to 9), because of the negative charge of the ssDNA drags the complex to the positive electrode. In lane 11, Qdots mixed ssDNA in the absence of the crosslinker reagent have similar mobility than pure qdots (line 1). ***B)*** STV-qdots modified with a nuclear localization sequence exhibit a decreased electrophoretic mobility as the number of NLS per qdot increase, because the NLS peptide is positively charged (lane 1 to 8). Lane 9-10 indicate that excess of biotin can reverse the NLS binding to the qdots.

3. The use of qdots in biodetection schemes

In the past years, we have worked with two types of functionalized qdots: single stranded DNA modified qdots for pathogen detection and peptides modified qdots for the targeting in the nucleus of living cells. The rest of the paragraph gives a brief overview of the experiments done.

3.a DNA-Qdots for pathogen detection.

In collaboration with Fanqing Chen from the Microarray Laboratory of LBNL, we have shown that DNA-qdots conjugates can detect single base polymorphisms (SNP) of genomic DNA in 10 minutes, at room temperature, with signal to noise ratio above 10 using cDNA microarray plateforms. This means that our DNA-qdots probes are able to detect and distinguish a modification of one base in any gene. For instance, we looked at gene p53 on chromosome 17, in which one single base change is observed in more than 50% of all cancers. Our DNA-qdot probes can detect a single base change out of 1179 bases composing p53 in a few minutes. This high selectivity extends not only to the detection of cancerous mutations, such as p53 on chromosome 17, but to the detection of viral and bacterial pathogens. For instance, DNA-qdot probes have been able to specifically detect the presence of the hepatitis B and hepatitis C virus genes among a background of other genes in a few minutes.

This very fast selectivity has to be associated with the sensitivity of the DNA-qdot probes. Unfortunately, the sensitivity is around nanomolar concentrations. Typical dyes, such as Cy3, are 100-1000 fold more sensitive with our plateform. Such reduced sensitivity prevents so far a wider range of applications of qdots in the screening for viral or bacterial pathogens.

The reasons for this poor sensitivity are still partially unclear. However, we have to keep in mind that in the current format of our assay, an estimated 10^9 dye molecules contribute to the detection signal, while the number of DNA-qdots that can hybridize on the same spot is at least 10 times smaller for steric hindrances reasons (Fig.3a-b). Current excitation/detection platforms are not sensitive enough. A possible solution to this problem is to scale down the dimensions of the array to the nanometer level and use stage scanning confocal microscopes with laser excitation to reach higher sensitivity. Indeed, we have shown with such setup that we can detect 4 colors of DNA-qdots in parallel when they hybridize onto a pattern of 8x8 μm^2 where the complementary DNA strand is attached. In that format, an estimated 10^5 particles contribute to the signal and the sensitivity of the qdot technology become comparable of that of the dyes (Fig.3c).

For even smaller features, such as at the nanometer scale, we believe that the qdot technology will outperform the dyes, because the much higher photochemical stability of the qdots generates in total more photons than a typical dye. The only problem so far is that the current printing technology for the fabrication of the microarray has not reached the nanolevel and therefore nanoarrays require expensive and slow production methods, such as nanolitogrphy.

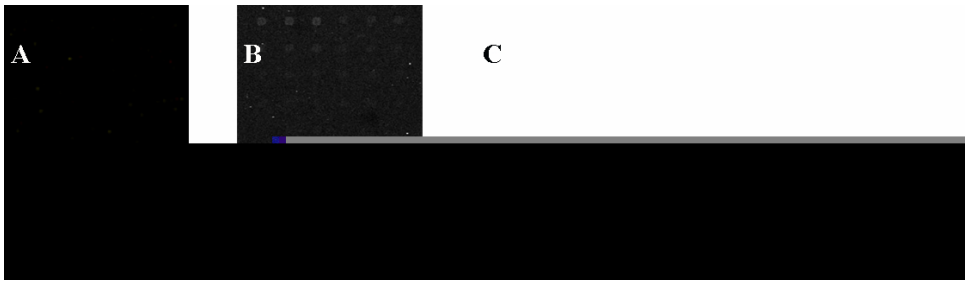

Figure 3. Detection of viral pathogen and single nucleotide mutations using ssDNA-qdots. ***A)*** Red qdots are programmed to recognized part of a gene specific to the hepatitis B virus (HBV), while yellow qdots are programmed to recognized part of a gene specific to the hepatitis C virus (HCV). When red and yellow qdots are mixed in a solution and dropped on a glass slide, each qdots recognize its target. Here, the targets are microprinted viral genomic sequences. Each sequence is printed in a 6-fold copy in a line. The array contains 10 different genes (i.e. 10 printed lines), but only the lines corresponding to HBV and HCV show up upon hybridization with the corresponding color code (red=HBV, yellow=HCV). ***B)*** ss-DNA qdots are exposed to part of gene p53, 25 bases long. The array contains 12 different genes (printed in a 3-fold copy in one line), some of them having only one base difference compared to the wild-type p53. Upon hybridization, ss-DNA qdots recognize only the perfect complement within a few minutes, at room temperature. Genes with 1 base mutations are not recognized***. C)*** Four colors of qdots, each with a different ss-DNA sequence, are mixed in a solution and are sorted upon hybridization on square gold pads. The size of the receptor site is 8 μm, and the number of qdots contributing to the signal is about 10^5. At this level, sensitivity of qdots becomes comparable to the sensitivity of dyes. For smaller features array, i.e. nanoarray, the qdot technology may well outperform the dye technology.
Spots sizes: A-B) 100 μm, C) 8 μm.

3.b NLS-Qdots for targeting the nucleus of living cells.

For a genomic analysis, one can looked at cells either in a cDNA array format, where the DNA of the cell is extracted and printed on a glass slide, or directly in-vivo. In this latter case, one needs to probe the interior of the cell nuclei while still maintaining the cell alive. In addition, the cell nucleus is a desirable target because numerous nuclear proteins actively participate in critical cellular processes such as DNA replication and recombination, RNA transcription, DNA damage and repair, genomic alterations and cell cycle control. In collaboration with Fanqing Chen, from the Life Science Division of Lawrence Berkeley Laboratory (LBL), we are interested in probing and monitoring the DNA damage and repair process upon ionization radiation. We would like to follow "life" the concentration upraise of some proteins, and their migration from the cytoplasm to the cell nucleus. The difficulty of monitoring the pathway of proteins using regular dyes comes essentially from two facts. First, the dyes photobleach too quickly and second, photobleaching is associated with the creation of free radicals that poison and therefore kill the cells. The use of qdots may solve the problem, because qdots are photochemically very stable and when they are embedded in a silica shell they are non-toxic to the cells. Indeed, cells can survive and divide for months even with silanized qdots in their interior.

In general, it has been proven difficult to target the nucleus of cells with qdots, because the nuclear pore size is about 25-30 nm and the solubilization schemes to make the qdots

water-soluble produce bulky compounds. We have succeeded in that (Fig.4). For that purpose, we have modified the surface of the qdots with a positively charged peptide sequences, called NLS for nuclear localization sequence.

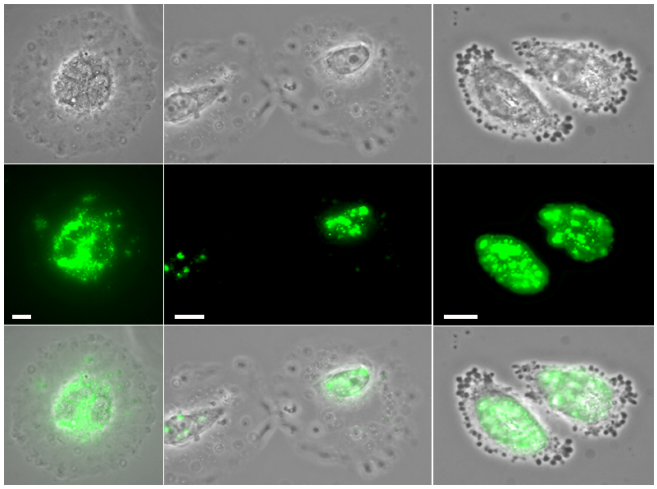

Figure 4. Labeling of nucleus of HeLa cells using qdots modified with SV40, a nuclear localization sequence. The qdots probes are introduced in the cells by electroporation. Cells stained with qdots maintain the ability to divide and can survive for months. Scale bars: 10 μm.

In a second step, living cells are transfected with NLS-Qdots by electroporation. NLS-qdots end up in two zones of the cells. In the majority of cases, the NLS-qdots end up in the perinuclear region of the cell, i.e. close to the nuclear membrane but outside the nucleus. In about the 15% of cases however, NLS-Qdots end up in the cell nucleus. We believe that the NLS-qdot complexes are about the size of the pores of the nucleus, and therefore a few of them enter. We can reduce the overall size of the complexes by linking the NLS directly onto the qdot instead of using a biotin-strepavidin bridge as we are doing now (Fig.1-H). This will result in a 5-7 nm reduction in size and should improve the efficiency of the nuclear targeting.

3.c Qdots for monitoring biological processes in living cells.

In late 2003, a team of researcher from France led by Maxime Dahan succeeded in following the diffusion of a neurotransmitter molecule, glycine, from one neuron to another during more than 20 minutes. In this case, glycine was linked to a qdot and the movement of the glycine was monitored by looking at the fluorescence of the qdot every 100 ms for 20 minutes. Such novel capability of following biomolecules while they "rock-and-roll" towards their receptors opens a vast new field of opportunities in biology.

In Livermore, along with our colleagues from LBL, we have explored the possibility to follow NLS-qdots when they try to enter the nucleus of a living cell. As a matter of fact, our setup is quite rudimentary so far and consists of an epifluorescence microscope with a digital camera that can collect one picture every 15 seconds. We collected pictures for

one hour. The resulting movie shows the jiggling of the NLS-qdots when they try to enter the cells nucleus and is illustrated in figure 5 for three different complexes of dots.

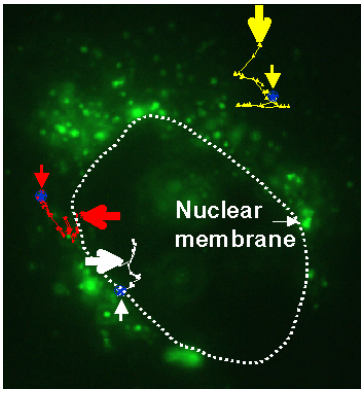

Figure 5. Trafficking of NLS-qdots in living cells. After electroporation, qdots migrate towards the cell nucleus where they accumulate. By monitoring the same region for an extended period of time (>15 min), we can observe the movements of the dots. Three examples are shown in the image. The position of the qdots at the beginning of observation is marked with a blue star and a small arrow. After 15 minutes, the qdot positions are indicated by the bold arrow. The path followed by each dot is indicated also. The qdots on the top of the image (yellow trail) diffuse around the nucleus but never come in close contact with the nuclear membrane. The other two qdots come in direct contact with the nuclear membrane, and seem to enter the nucleus. Integration time is 500 ms per frame. Scale bar: 2 µm.

A further analysis of the process will require a more advanced setup, as the one used by the French group. However, our preliminary experiments showed the possibility to target organelles in living cells and following the pathway of individual biomolecules while they migrate to their receptor sites. The monitoring of the trafficking inside living cells calls for a close collaboration between biologists and physico-chemists which we hope to establish in Livermore.

4. Our current activity at LLNL

The previous chapter gave an overview of the capabilities provided by the use of qdots as biolabels. However, to a large extend, many properties of the qdot complexes are not known. Open issues include the following points: do qdots have large term toxicity, can they be used in in-vivo FRET experiments as replacements for dyes, and if yes how can we exploit these properties at the single molecule level. This list of open issues is certainly not complete though.

As a start, we want to orient our research in four broad directions, which from my perspective, have a broad overlap. These directions are:

1. Study of toxicity of nanocrystals with toxicogenomic profiling using microarrays.
2. Synthesis and characterization of new nanoprobes including new ones with dual functionality, for instance fluorescent probes that are active for magnetic resonance imaging.
3. Study of photophysical properties of biomodified-qdots at the single-molecule level.
4. Use of biomodified-qdots for targeting organelles in living cells (Mitochondria, siRNA, etc..)

These studies will be conducted in collaborations with others groups. Our collaborators include:

Dr. Fanqing Chen, from the Life Science Division of LBL.
Dr. Thomas Huser, LLNL.
Prof. Denise Krol, UC Davis.
+ other biologists we are trying to convince.
+ all the people who help us and support us everyday.

If you are interested in these experiments and would like to have more details, please feel free to send us an email.